Localization by Photoaffinity Labeling of Natriuretic Peptide Receptor-A Binding Domain†

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ABSTRACT: A portion of the ligand binding domain for atrial natriuretic peptide (ANP) was identified as an affinity cross-linked proteolytic fragment of bovine adrenal natriuretic peptide receptor type-A (NPR-A). Affinity purified NPR-A was UV-cross-linked to the amino terminus of ^{125}I-[Tyr^2] rat ANP-(2−27). A chymotryptic fragment of the affinity labeled NPR-A was isolated by chromatography and electrophoresis. This fragment yielded a major microsequence corresponding to a region from Met^{173} to Phe^{188} of the receptor extracellular domain and containing one N-glycosylation site at Asn^{180}. Bovine NPR-A receptor was then cross-linked to the carboxy terminus of the highly efficient photoaffinity derivative ^{125}I-[Tyr^{18},Bpa^{27}] rat ANP(1−27). Proteolysis of the affinity labeled NPR-A with cyanogen bromide and trypsin produced radiolabeled and glycosylated fragments of size 15 and 9 kDa, respectively, which contained the epitope Ile^{181}-Phe^{188} (CS328) and which were detectable by immunoprecipitation with a monospecific polyclonal antibody against CS328. Proteolysis with cyanogen bromide followed by Glu-C produced a shorter photolabeled 6 kDa fragment which was not immunoprecipitable by anti-CS328 antibody and which was not glycosylated. The results lead to the identification of the short segment Asp^{191}-Arg^{198} as the site of covalent binding of [Tyr^{18},Bpa^{27}] rat ANP(1−27). This hydrophilic region is adjacent to the epitope Ile^{181}-Phe^{188} and to the glycosylation site Asn^{180}. It displays the species variability and the high surface probability expected for a portion of the binding domain of NPR-A in contact with ANP.

Natriuretic peptides are a family of closely related factors which exert profound regulatory effects on cardiovascular homeostasis (Genest & Cantin, 1988; Inagami, 1989; Brenner et al., 1990). Natriuretic peptide type-A (ANP)[1] is the main cardiac natriuretic factor synthesized in atrial cardiomyocytes. Its role as a major cardiovascular factor opposing the renin−angiotensin system has been documented in the kidney, the adrenal, and the vasculature and was recently confirmed by gene knockout experiments (John et al., 1995). BNP shares most of the biological properties of ANP while CNP which can be synthesized in endothelial cells is predominantly vasorelaxant (Aburaya et al., 1989; Stingo et al., 1992).

NPR-A and NPR-B receptors are selective for ANP and CNP, respectively (Chinkers et al., 1989, 1991; Lowe et al., 1989; Koller et al., 1991). These two receptor classes belong to a growing family of membrane-bound guanylate cyclase proteins (Fülle et al, 1995). NPR-A and NPR-B are noncovalent homooligomers characterized by four domains: the extracellular domain which provides the binding site for the activating peptide; the transmembrane domain; the kinase homology domain which binds ATP and which modulates receptor activation; and the guanylate cyclase catalytic domain (Chinkers & Wilson, 1992; Lowe, 1993). Activation of NPR-A appears to require dimer or oligomer formation (Chinkers & Wilson, 1992; Lowe, 1993). However, in contrast with growth factors and cytokines, natriuretic peptide binding has not been shown to induce dimerization of their receptor. NPR-C receptors are structurally and functionally distinct (Fuller et al., 1988). They are constituted of disulfide-bridged dimers with short cytoplasmic domain and devoid of guanylate cyclase activity. Receptor isoforms of NPR-A (Tallerico-Melnyk et al., 1995), NPR-B (Okyama et al., 1992), and NPR-C (Mizuno et al., 1993) have been reported but their significance remains to be determined.

NPR-C receptor extracellular domain displays five cysteines (or six for NPR-C6 isoform). The first four cysteines are sequentially paired disulfide-bridged and form 29-residue and 49-residue intrachain loops while the last one (or two) is involved in covalent homodimer formation (Itakura et al., 1994; Stults et al., 1994). Intrachain loops in NPR-C appear to be required for maintaining natriuretic peptide binding function while covalent homodimer formation can be prevented apparently without changing peptide binding (Itakura et al., 1994). Three of the four potential N-glycosylation sites are actually glycosylated (Stults et al., 1994). Site-

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[1] Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR-A, type-A natriuretic peptide receptor; NPR-B, type-B natriuretic peptide receptor; NPR-C, type-C natriuretic peptide receptor; BPA-ANP, [Tyr^{18},*p*-benzoyl-Phe^{27}]rat ANP(1−27); Tyr^2 ANP, [Tyr^2] rat ANP(2−27); CS328, peptide YITVDHLEF; PABA, *p*-aminobenzoic acid; EDTA, ethylenediaminetetraacetic acid, sodium salt; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CNBr, cyanogen bromide; BSA, bovine serum albumin.

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directed mutagenesis has identified several amino acid residues important for receptor function. His^{104} and Trp^{105} which are highly conserved among all natriuretic peptide receptors appear to be either constituents or determinants of the peptide binding domain (Iwashina et al., 1994). In addition, variable residues 188 (Ile or Ala) and 205 (Tyr, His or Asn) are also involved in determining species differences of ligand binding specificity (Engel et al., 1994, 1995).

For NPR-A and NPR-B receptors extracellular domain disulfide-bridged intrachains have not yet been demonstrated but conservation of the first four cysteine positions in NPR-A and of three positions for NPR-B suggests the existence of equivalent loops (Itakura et al., 1994; Stults et al., 1994). Site-directed mutagenesis has identified Leu^{364} and Glu^{332} as potentially important determinants of natriuretic peptide binding specificity in NPR-A and NPR-B, respectively (Duda et al., 1995). Five of the seven consensus sites for N-glycosylation of NPR-B are substituted, and Asn^{24} glycosylation is crucial for proper receptor function (Fenrick et al., 1996).

Covalent labeling of growth factor receptors, e.g., EGF and insulin with their activating peptide, has been used to delineate important regions of the peptide binding domain of their receptor (Lax et al., 1988; Wu et al., 1990; Yip et al., 1988; Waugh et al., 1989). The experimental approach involved proteolytic cleavage of ligand cross-linked receptor using agents, e.g., CNBr, Glu-C, or other proteases. The proteolytic fragment covalently attached to the ligand was identified by a combination of methods including immunodetection of a characterized epitope in the extracellular domain. In few cases the specific amino acid residue covalently attached to the ligand could be unambiguously identified, but more often a segment of the linear sequence of the receptor was localized within the peptide binding domain.

We have previously shown that underivatized ANP can be UV-cross-linked through its amino-terminal end with an efficiency equivalent to that of azido-derivatives of natriuretic peptides (Larose et al., 1990). We have previously developed the highly efficient derivative [Tyr^{18},Bpa^{27}] rat ANP(1−27) containing the photosensitive derivative benzoylphenylalanine (Bpa) (McNicoll et al., 1992). We have also modified this photoaffinity derivative by including an azido derivative at its amino-terminal end (Rondeau et al., 1995). Application of this bifunctional photosensitive ANP analog to the study of NPR-A provided evidence that one molecule of ANP is binding per NPR-A receptor dimer and that the amino- and the carboxy-terminal ends of ANP might be adjacent to distinct subunits of the receptor dimer (Rondeau et al., 1995).

In this study, we have isolated and characterized ANP-labeled proteolytic fragments of bovine adrenal NPR-A using two ANP derivatives: [Tyr^2] rat ANP(2−27) which can be UV-cross-linked through its amino terminal and [Tyr^{18},Bpa^{27}] rat ANP (1−27) which is efficiently incorporated through its carboxy terminal. Alignment of the overlapping sequence of proteolytic fragments with the primary amino acid sequence of NPR-A enabled the identification of a short region in contact with ANP.

EXPERIMENTAL PROCEDURES

Materials. BPA-ANP and Tyr^2 ANP were synthesized by IAF Biochem as previously described (McNicoll et al., 1992). PABA, TPCK-treated trypsin, chymotrypsin, pepstatin A, bovine serum albumin (BSA), glutaraldehyde, and peptide molecular weight markers were from Sigma. Leupeptin, pefabloc, aprotinin, endoproteinase Glu-C, endoglycosidase F/N-Glycosidase F, sodium dodecyl sulfate, glycine, tricine, and acrylamide were from Boehringer Mannheim. Iodo-beads were from Pierce Chemical Co., and carrier free $Na^{125}I$ was from Amersham. Electrophoresis molecular weight markers were from Bio-Rad. LH-60 agarose and Protein A Sepharose were from Pharmacia. Centricon-100 ultrafiltration units wer from Millipore. All other reagents were from commercial sources.

Preparation of Membranes. Bovine adrenal zona glomerulosa membranes were prepared as previously described (McNicoll et al., 1992) and stored at −70 °C until used.

Iodination of BPA-ANP and Tyr^2 ANP. Briefly, the peptide (3 nmol) was incubated with 1 mCi of $Na^{125}I$ in the presence of two Iodo-Beads, and the monoiodinated derivative was purified on reverse-phase Vydac C18 column as previously described (McNicoll et al., 1992). The specific activity of the monoiodinated derivative was ∼2000 Ci/mmol.

Purification of NPR-A and Photolabeling with ^{125}I-Tyr^2 ANP. Bovine zona glomerulosa membrane receptors were solubilized and purified to homogeneity by affinity chromatography on ANF-agarose, followed by steric exclusion Superose 6 HPLC column (Meloche et al., 1988). One part of that preparation was digested with trypsin at 25 °C according to methods described under *Proteolytic Cleavage* and purified by HPLC on reverse-phase Vydac C18 column using a gradient of 15−55% of acetonitrile in 0.1% TFA. Peaks detected by absorbance at 214 nm were collected manually and microsequenced. The other part of pure receptor preparation was photolabeled with ^{125}I-Tyr^2ANF using malondialdehyde generated by UV irradiation (Larose et al., 1990). The photolabeled protein was cleaved with chymotrypsin as detailed in section on proteolytic cleavage and loaded on 10% SDS−PAGE according to Schägger and Jagow (1987). The radioactive band (14 kDa) was eluted and chromatographed on LH-60 steric exclusion gel (22 mL swollen gel) in 21% formic acid, 66% ethanol, and 13% H_2O according to modifications of methods described (Gerbers et al., 1979). The fractions containing the photolabeled fragment were pooled and microsequenced.

Microsequencing of Proteolytic Fragments. Automated Edman degradation was performed using an Applied Biosystem Model 477A liquid-pulse protein sequence, and amino acid phenylthiohydantoin derivatives were identified according to the protocol recommended by the manufacturer on a Model 120A HPLC system.

CS328 Antibody Production and Immunoprecipitation. The peptide YITVDHLEF (CS328) corresponding to the carboxy terminal of a microsequenced chymotryptic fragment of NPR-A was coupled to BSA using glutaraldehyde, and 100 μg of conjugate was injected subcutaneously to rabbits. Antibodies obtained after three boosts were characterized (Rondeau et al., 1995). For immunoprecipitation samples were incubated with anti-CS328 antibody diluted 1:40 in a final volume of 100 μL containing 50 mM sodium phosphate and 1% Triton X-100, pH 7.4. After incubation at 4 °C for 18 h, 20 μL of protein A Sepharose was added, and the incubation was continued for 4 h at 4 °C. The gel was then washed four times with 10 volumes of incubation buffer,

and the radioactive fragments were eluted in sample buffer, at 100 °C for 3 min.

Receptor Binding and Photolabeling with ^{125}I BPA-ANP. Membranes were incubated in the dark in the presence of 100 pM ^{125}I BPA-ANP in 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 5 mM $MnCl_2$, and 0.5% BSA (buffer A). After 90 min at 22 °C, the proteins were pelleted at 40000*g* for 30 min and resuspended in 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 5 mM $MnCl_2$, and 0.1 mM PABA (buffer B). Following irradiation with UV lamps (365 nm) for 15 min at 4 °C, the membranes were centrifuged and denatured at 100 °C for 3 min in sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue). Buffers A and B were degassed under vacuum, sparged with nitrogen, and used in capped tubes in order to minimize lipid peroxydation.

Preparative Electrophoresis. The photolabeled receptor was purified by PAGE on a 3 mm thick 7.5% SDS gel (Laemmli, 1970). Bands corresponding to 130 kDa proteins were sliced into $1mm^3$ fragments and eluted twice in five volumes of 0.1% SDS/50 mM NH_4HCO_3. Proteins were concentrated on Centricon-100 up to 2 mg/mL.

Reduction and Alkylation. Reduction was performed at room temperature, with 5 mM DTT in 50 mM ammonium bicarbonate for 90 min under nitrogen. After addition of 10 mM iodoacetic acid, the incubation was continued in the dark for an other 60 min under nitrogen.

Proteolytic Cleavage. Prior to cleavage at methionines the receptor preparation was reduced by incubation with 100 mM β-mercaptoethanol for 4 h followed by dialysis. CNBr cleavage was performed in 70% formic acid for 20 h at 22 °C with CNBr at 1 mg/mg of protein. The digestion medium was diluted 10-fold with water, and the reaction mixture was freeze-dried. Trypsin digestion was performed using 50 μg of enzyme/mg of protein in 50 mM NH_4HCO_3, 2 mM $CaCl_2$, pH 8.5, for 18 h at 37 °C. Glu-C treatment was done with 50 μg of enzyme/mg of protein in 50 mM NH_4HCO_3, pH 7.8, for 18 h at 22 °C. Chymotryptic fragments were obtained by incubation with TPCK-treated chymotrypsin at 50 μg/mg of proteins in 50 mM NH_4HCO_3 and 2 mM $CaCl_2$, pH 8.0, for 18 h at 25 °C. Deglycosylation was performed with N-glycosidase F at 50 milliunits/μg of protein in 250 mM sodium phosphate buffer, pH 7.5, 10 mM mercaptoethanol, 10 mM EDTA, and 0.6% Triton-X100 for 24 h at 25 °C.

Electrophoresis and Western Blotting. SDS−PAGE electrophoresis was done with Tricine as the trailing ion according to Schägger and Jagow (1987). The fragments separated on gel were transferred on Nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol, using LKB Novablot system, and radiolabeled fragments were detected by autoradiography.

RESULTS

The experiments presented aimed at localizing the binding domain for ANP in the extracellular portion of NPR-A. Photoaffinity labeling of the receptor protein and characterization of its proteolytic fragments were attempted in order to directly identify a primary sequence region involved in ligand−receptor interaction. Earlier results indicated that lectins could interfere with ANP binding, suggesting that a glycosylation site might be in the vicinity of the ANP binding

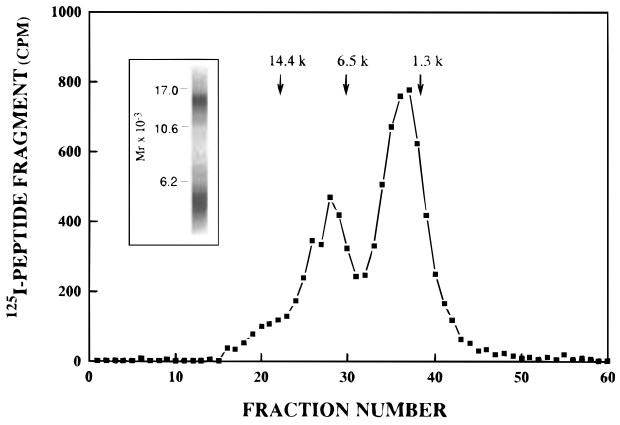

FIGURE 1: Isolation of ANP-photolabeled NPR-A chymotryptic fragment by electrophoresis and size exclusion chromatography. Affinity-purified bovine adrenal NPR-A was photolabeled with ^{125}I-Tyr^2-ANP by UV-cross-linking. The amino terminus of the peptide was covalently attached to a free amino group of NPR-A by reacting with malondialdehyde generated by lipid peroxydation. The photolabeled receptor was proteolyzed with chymotrypsin, and the fragment was first isolated by 10% SDS−PAGE (inset) and then by LH-60 size exclusion chromatography in ethanol/acid mobile phase.

domain (Liu et al., 1989). More recent results also showed that a single molecule of ANP was binding to the NPR-A dimer and that the amino- and the carboxy-terminal ends of ANP could be interacting with distinct subunits of the receptor dimer (Rondeau et al., 1995).

We first attempted covalent attachment of ANP to NPR-A by using a method based on the UV-generated cross-linking agent malondialdehyde which covalently attaches ^{125}I-ANP through its amino terminal to primary amines of the receptor protein by forming a Schiff base bond (Larose et al., 1990; Manwaring & Csallany, 1988; Chio & Tappel, 1969; King, 1966). We modified the ANP peptide to [Tyr^2] rat ANP-(2−27) so that the radiolabeled tyrosine positioned at the amino terminus could be retained in a chymotryptic fragment of the ANP-cross-linked receptor.

Bovine adrenal zona glomerulosa NPR-A protein was purified by affinity chromatography using Affi-Gel-ANP (Meloche et al., 1988). The affinity-purified receptor was UV-cross-linked with ^{125}I-[Tyr^2] rat ANP(2−27), and the covalent ligand−receptor complex was cleaved with chymotrypsin. The radiolabeled fragment was isolated by SDS−PAGE electrophoresis and by size exclusion chromatography on LH-60. Figure 1 shows that a main 7 kDa peak was obtained after separation from free ^{125}I-Tyr^2 ANP. Following treatment with N-glycosidase F, this fragment migrated on SDS−PAGE down with the elution front corresponding to a small molecular weight <2 kDa, indicating that it was glycosylated (data not shown).

Microsequencing of this chymotryptic fragment provided a major sequence MXVXDXLXITVDXLEF corresponding to region Met^{173}-Phe^{188} of the NPR-A receptor sequence (Figure 5). As expected, arginines 174, 176, and 178 were not clearly detected, presumably because of their modification during UV-cross-linking (King, 1966). This interpretation is supported by the observation of incomplete proteolysis of NPR-A receptor protein with trypsin following UV-cross-linking (data not shown; King, 1966). Asn^{180} was not detected consistent with its expected N-glycosylation. His^{185} was not positively identified. The two positions Asp^{184} and

Leu[186] which display species variability were identical to those for human NPR-A sequence (Figure 5; Schoenfeld et al., 1995). This result is consistent with those obtained by extensive microsequencing of bovine NPR-A tryptic fragments which provided sequences encompassing nearly 20% of the protein (data not shown). Comparison of bovine NPR-A tryptic fragment sequences with those reported for other species indicated that bovine NPR-A displayed higher overall identity homology with human (97%) than with rat (95%) or mouse (92%) NPR-A, the difference being even more pronounced (98% versus 94% and 89%) when only the extracellular domains were considered (data not shown). Therefore, human NPR-A sequence was currently used as a reference for the following studies of bovine NPR-A.

Microsequencing of the chymotryptic fragment also provided a minor sequence XYPPPDVPXGF..., corresponding to a region beginning at Gly[414] and possibly ending within the transmembrane domain. This minor sequence does not contain a potential glycosylation site. Although both sequences were initially considered, the major sequence Met[173]-Phe[188] was selected and used for raising monospecific polyclonal antibodies against its carboxy-terminal portion Ile[181]-Phe[188] (CS328).

In order to further document a proteolytic fragment obtained from ANP binding domain, we used the high efficiency photoaffinity derivative [Tyr[18],Bpa[27]] rat ANP-(1−27) which can be cross-linked to the receptor protein through its carboxy terminal (McNicoll et al., 1992). This derivative is based on the photosensitive amino acid Bpa which efficiently forms a stable covalent bond with electron-rich C−H bonds, e.g., backbone C_α−H, C_β−H of valine, C_γ−H of leucine, and CH_2 groups adjacent to heteroatoms in lysine, arginine, and methionine (Dorman & Prestwich, 1994). Positioning of the photosensitive derivative at the carboxy-terminal end of ANP appeared optimal because this region is critical for high affinity binding to NPR-A, therefore favoring attachment of ANP to residues within the ligand binding pocket. Additional cross-linking of the radioligand through its amino terminal due to lipid peroxydation and production of malondialdehyde was prevented by performing UV photoactivation in N_2-saturated buffer in the presence of the radical scavenger PABA.

Photolabeled NPR-A was cleaved with cyanogen bromide and the presence in the proteolytic fragments of the CS328 epitope was studied by immunoprecipitation with anti-CS328 antibody. Cyanogen bromide cleavage of photolabeled NPR-A yielded a major fragment at 15 kDa with additional bands at 40, 32, and 12 kDa (Figure 2A). This proteolytic cleavage profile was highly reproducible. Pretreatment of photolabeled NPR-A with the reducing agent DTT did not lead to more complete cleavage suggesting that larger fragments were not due to oxydation of methionines (data not shown). Photolabeled cyanogen bromide fragments were also immunoprecipitable by an anti-CS328 antibody indicating that they contained the CS328 epitope (Figure 2B). These results suggested that the major 15 kDa cyanogen bromide fragment might correspond to the region of NPR-A from Arg[174] to Met[223], assuming poor cleavage at Met[203] or its replacement with Val[203] as in rat and mouse NPR-A (Figure 5).

Further proteolysis with trypsin of CNBr fragments of photolabeled NPR-A led to a major fragment of 10 kDa (Figure 2A). Immunoprecipitation of this photolabeled

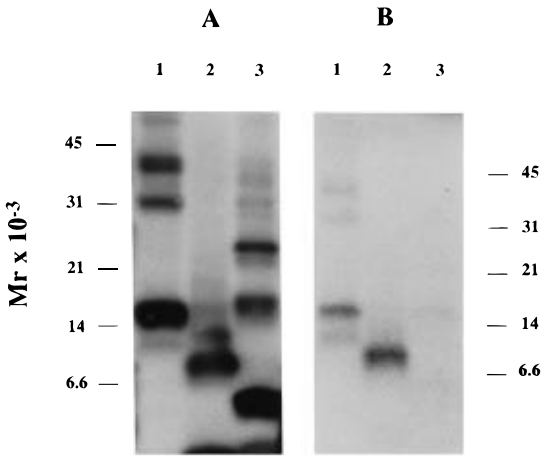

FIGURE 2: Separation of proteolytic fragments of BPA-ANP photolabeled NPR-A receptor. Bovine adrenal NPR-A was photolabeled with ^{125}I-BPA-ANP then proteolyzed with CNBr alone (lanes 1), CNBr followed by trypsin (lanes 2), or CNBr followed by Glu-C (lanes 3). Aliquots from the proteolysis reacting media were either immediately prepared for SDS−PAGE (panel A) or immunoprecipitated with anti-CS328 antiserum before electrophoresis (panel B).

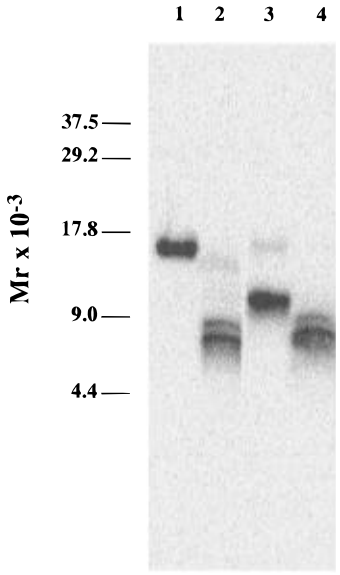

FIGURE 3: Effect of deglycosylation on proteolytic fragments of BPA-ANP photolabeled NPR-A receptor. Bovine adrenal NPR-A was photolabeled with ^{125}I-BPA-ANP then cleaved with CNBr. The proteolytic fragments were separated on SDS−PAGE, and the major 15 kDa band was eluted then treated with none (lanes 1 and 3) or Glu-C (lanes 2 and 4). In addition, samples show in lanes 3 and 4 were further treated with endoglycosidase F. The gel was electrotransferred to nitrocellulose and radioactive bands were detected by PhosphorImager.

tryptic fragment also indicated that it contained the epitope Ile[181]-Phe[188] (Figure 2B). The major tryptic fragment could be interpreted as ranging from Val[175] to Arg[198] of NPR-A assuming poor cleavage at Arg[176] and Arg[178] due to neighbouring Asp[177] and with cleavage of covalently attached BPA-ANP after Arg[14] (Figure 5).

On the other hand subsequent treatment with Glu-C of CNBr fragments lead to a major 6 kDa band together with additional 16 and 25 kDa bands (Figure 2A). No radioactive band could be detected after immunoprecipitation of the fragments obtained with CNBr together with Glu-C (Figure 2B). The major 6 kDa band is compatible with the unglycosylated fragment Arg[191] to Met[223] which does not

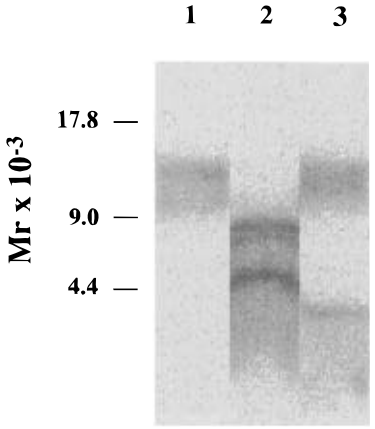

FIGURE 4: Properties of tryptic fragments of BPA-ANP photolabeled NPR-A receptor. Bovine adrenal NPR-A was photolabeled with ^{125}I-BPA-ANP and then cleaved with CNBr. The proteolytic fragments were separated on SDS-PAGE and the major 15 kDa band was eluted then treated with trypsin alone (lane 1), trypsin and endoglycosidase F (lane 2), or both trypsin and Glu-C (lane 3). The gel was electrotransferred to nitrocellulose and radioactive bands were detected by PhosphorImager. In the experiment shown, proteolysis with Glu-C was incomplete.

contain the CS238 epitope (Figure 5). Alternatively, Glu-C might have altered the CS328 epitope by cleaving at Glu^{187} resulting in fragment Met^{173} to Glu^{187}.

The alternative interpretations of the CNBr with Glu-C fragments were tested by studying the effect of deglycosylation on proteolytic fragments of photolabeled NPR-A. The 15 kDa cyanogen bromide fragment was first isolated by SDS−PAGE from other minor fragments and then subjected to either Glu-C or trypsin treatment or to both and then with N-glycosidase. As documented above (Figure 2) treatment of the 15 kDa CNBr fragment with Glu-C reduced its size to 6 kDa (Figure 3). This fragment was not glycosylated as judged by its insensitivity to N-glycosidase (Figure 3). The fragment resulting from both CNBr and Glu-C treatments could be best interpreted as corresponding to region Asp^{191} to Met^{223} which is not glycosylated (Figure 5). Cleavage of the 15 kDa CNBr fragment with trypsin lead to a 10 kDa fragment which could be further reduced to 5 kDa by deglycosylation and was therefore glycosylated (Figure 4). Proteolysis of the 15 kDa cyanogen bromide fragment with both trypsin and Glu-C resulted in a small 2.5 kDa fragment (Figure 4). This small photolabeled fragment is compatible with region Asp^{191} to Arg^{198} and corresponds to the shortest photolabeled NPR-A fragment documented.

DISCUSSION

Photochemistry of benzophenone photophore has been widely applied to determining ligand binding sites (Dorman & Prestwich, 1995). These derivatizes, e.g., benzoylphenylalanine (Bpa), are highly efficient and the required geometry of the hydrogen abstraction and recombination steps limits the reaction to a sphere of 3.1 Å radius, therefore restricting the number of potential acceptor sites to residues in close contact. Modeling of ANP three-dimensional structure based on NMR studies of derivatives indicates that its carboxy-terminal end is looping back toward the important intraloop region Phe^{8}-Ile^{15} so that residue Arg^{27} is juxtaposed to this region (Fairbrother et al., 1994). Replacement of Arg^{27} by Bpa^{27} in ANP is therefore strategic and properly positions this photoreactive residue for optimal probing of the ligand binding pocket of NPR-A.

Interestingly, our results document that the proximal region Met^{173}-Phe^{188} of the NPR-A protein is also probed by covalent cross-linking of ANP through its amino terminus. This region is immediately adjacent to Asp^{191}-Arg^{198} of the receptor which is in contact with the carboxy terminus of ANP. This suggests that the amino- and the carboxy-terminal ends of ANP are in contact with closely positioned regions of NPR-A. These results appear contradictory to those on modeling of ANP which suggest that the amino- and the carboxy-terminal ends of the peptide are located on opposite sides of the molecule (Fairbrother et al., 1994). One possible interpretation could be that the amino- and the carboxy-terminal ends of ANP might be interfacing with distinct subunits of NPR-A homodimer, a hypothesis supported by previous results based on a bifunctional photoaf-

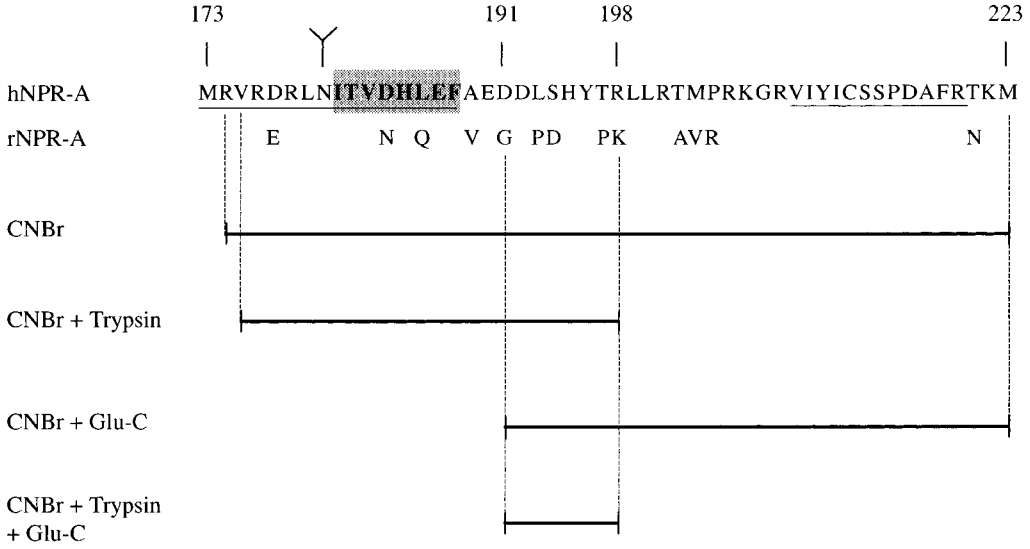

FIGURE 5: Localization of photolabeled proteolytic fragments in NPR-A. The human NPR-A sequence (hNPR-A) is shown together with rat NPR-A (rNPR-A). Underlined residues correspond to microsequenced proteolytic fragments from bovine NPR-A. The glycosylation site is indicated with a Y-shape pointer. The CS328 epitope is highlighted. The overlapping segment Asp^{191}-Arg^{198} common to fragments obtained with either CNBr and trypsin (glycosylated) or CNBr and Glu-C (unglycosylated) defines the region of covalent attachment of BPA-ANP.

finity derivative of ANP (Rondeau et al., 1995). This ligand binding conformation is analogous to that for GH binding to its dimeric receptor (de Vos et al., 1992). Crystallography of GH−GHR receptor complex reveals that each subunit of the receptor homodimer is presenting the same binding pocket for contacting opposite sides of the single GH molecule bound to the receptor dimer (de Vos et al., 1992). This sandwiched contacting appears to be important for inducing receptor dimerization and activation. GH analogs modified at residues determinant for one of the contacting sites failed to induce dimerization and lacked the activation properties of the natural hormone (Wells et al., 1993). In analogy with the GH receptor, homodimeric NPR-A might be binding natriuretic peptides by simultaneous interaction of both receptor subunits with opposite sides of the peptide. This simultaneous interaction might be required for high affinity binding characteristics insuring specificity of the receptor and for proper transmembrane activation of cytoplasmic guanylate cyclase domains of the receptor.

Our results obtained by photoaffinity of NPR-A can be compared with those obtained by site-directed mutagenesis of NPR-C. Engel et al. have documented important residues for natriuretic peptide binding to the disulfide-bridged NPR-C (Engel et al., 1994; Engel & Lowe, 1995). Residues Ile^{188} and Asn^{205} appear to be critical for determining species differences in the affinity of natriuretic peptide analogs (Engel et al., 1994; Engel & Lowe, 1995). These residues were putatively determinant for the successful development of natriuretic peptide analogs displaying very low affinity for human NPR-C but high affinity for human NPR-A (Cunningham et al., 1994). Residues Ile^{188} and Asn^{205} from NPR-C are localized in the vicinity of the region Asp^{191}-Arg^{198} that we documented by NPR-A photoaffinity labeling. Ile^{188} of NPR-C corresponds to His^{185} of NPR-A located within the epitope Ile^{181}-Phe^{188} (CS-328) of NPR-A. Asn^{205} of NPR-C corresponds to Thr^{202} located within four residues of the region Asp^{191}-Arg^{198} associated with the carboxy-terminal attachment of ANP to NPR-A. The similar localization of these residues determined by different methods, i.e., photoaffinity labeling and site-directed mutagenesis, suggests that this region is important for natriuretic peptide binding to both NPR-A and NPR-C. This ANP binding domain portion of NPR-A is also adjacent to glycosylation site Asn^{180} and is associated with a high probability of being at the protein surface. These results confirm earlier observations which suggested the presence of a glycosylation site in the proximity of ANP binding site (Liu et al., 1989). This region of NPR-A is also one of the most variable and might also contribute to species differences in NPR-A binding (Féthière & De Léan, 1991; Schoenfeld et al., 1995).

Site-directed mutagenesis of NPR-C based on highly conserved residues has provided evidence that residues His^{145}-Trp^{146} common to all natriuretic peptide receptors are critical for natriuretic peptide binding (Iwashina et al., 1994). Although not yet confirmed for other NPR, these residues might also be important for NPR-A ligand binding function. Whether these residues are directly contacting natriuretic peptides or are indirect determinants for proper ligand binding domain conformation is yet unknown. Other distinct residues Leu^{364} and Glu^{332} associated with the selectivity properties of NPR-A and NPR-B, respectively, have been also documented (Duda et al., 1995). Site-directed mutagenesis of those residues enable both ANP and CNP to potently activate either NPR-A or NPR-B mutants resulting in loss of specificity (Duda et al., 1995). Those residues are localized in a more conserved region than those reported in the present report and their role in directly or indirectly contributing to the ligand binding domain remains to be assessed. Contribution to the ligand binding domain of amino acid residues located in distant regions of NPR-A primary sequence is expected, and further studies will be required to assess their importance in determining the natriuretic peptide binding properties of dimeric natriuretic peptide receptors.

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